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Evaluation of Mutagenic Effects of Diesel Emissions: I. Tests for Heritable and Germ-Cell Effects in the Mouse

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16. ABSTRACT

The Environmental Protection Agency, under the Clean Air Act, is charged with the responsibility for regulating the emissions from new motor vehicles. In order to assess potential heritable effects of diesel exhaust, mice were exposed to whole diesel exhaust by inhalation and a number of genetic end points were studied. Exposure times varied from 5 to 10 weeks for the different groups. The diesel particulate concentration averaged 6 mg/m^3 during the exposure period of 8 hours per day and 7 days of the week. The results of all genetic assays in both sexes were negative; however, small but unequivocal effects on the reproductive performance of females of one strain were observed.

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EVALUATION OF MUTAGENIC EFFECTS OF DIESEL EMISSIONS I. TESTS FOR HERITABLE AND GERM-CELL EFFECTS IN THE MOUSE

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The Genetic Toxicology Division supported this study in order to determine whether or not whole diesel emissions produced a demonstrable effect in a battery of in vivo mammalian assays for heritable effects. The assays were chosen to detect several types of genetic end points after exposure by inhalation to whole diesel exhaust. Such an assessment helps to provide an adequate data base for future decisions by the Administrator regarding requirements for emission standards from mobile sources.

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ABSTRACT

In order to assess potential risk from heritable effects in human populations, mice were exposed by inhalation to whole diesel exhaust, and a number of genetic endpoints were studied. Exposure times in different groups varied from 5 to 10 weeks. In the maximally-exposed group, the total intake of diesel exhaust per mouse was about 85 times the 30-year (generation-length) intake by a person in an average U.S. environment.

The battery of assays was chosen to detect several types of genetic endpoints, namely, point mutations in males (specific-locus test), chromosome damage in males (dominant-lethal and heritable-translocation tests), and chromosome damage in females (dominant-lethal tests). Ancillary studies were carried out to look for direct reproductive damage in both sexes; thus, various parameters were used to assess reproductive performance in females, and histological analyses of germ-cell survival were done in males.

The results of all genetic assays in both sexes were negative. In the ancillary tests, small but unequivocal effects on the reproductive performance of females of one strain could be observed, consisting of a decrease in the number of ovulations and an increase in the interval between mating opportunity and copulation. There was no detectable effect of diesel exposure on the number and distribution of cell types in the testis.

The absence of genetic effects could indicate either that no active metabolites reached the gonads, or that the germcells have an efficient repair system against induction of mutational events by such metabolites. Thus, transmitted genetic effects appear not to be a major hazard from exposure to diesel exhaust. The findings reported must, however, not be used to draw any conclusions concerning possible risks to the exposed individual him/herself.

CONTENTS

Foreword iii
Abstract iv
Figures vi
Tables vii

1. Introduction 1
2. Overall Conclusions 2
3. General Procedure 3
4. Reports of individual experiments 5
   - Test for heritable point mutations in male mice 5
   - Test for induction of dominant lethals in male mice . . . 10
   - Test for induction of heritable translocations in male mice 13
   - Test for induction of genetic effects and oocyte killing in females 15
   - Test for dominant-lethal induction in female mice 18
   - Test for effects on spermatogonial survival in the mouse 24

FIGURES

Number		Page
1	Distribution of fertile females with respect to number of corpora lutea	21
2	Distribution of copulated females with respect to caging-copulation interval	21

TABLES

Number		Page
1	Results of specific-locus test, following exposure of male mice to diesel exhaust	7
2	Specific-locus results grouped by germ-cell stage	8
3	Test for induction of dominant-lethal mutations in male mice	11
4	Induction of heritable translocations in male mice with diesel exhaust.	14
5	Reproductive performance of female mice exposed to diesel exhaust	16
6	Mating performance and results of uterine dissections for exposed females and controls	19
7	Distribution of corpora lutea counts in exposed females and controls	20
8	Distribution of mating intervals in exposed females and controls	22
9	Average number of corpora lutea at different mating intervals	23
10	Mean number of spermatogonia and preleptotene spermatocytes observed after exposure to diesel exhaust for 5 and 10 weeks.	25

1. INTRODUCTION

Although non-mammalian and in vitro assays can provide evidence on presence or absence of mutagenicity, they have limitations in addressing the complexities associated with reproductive-cell targets in intact organisms. The assessment of risk from heritable damage must therefore utilize *in vivo* mammalian germline mutagenicity tests.

The effectiveness of inhaled whole diesel exhaust in inducing heritable effects in mammals was studied by a battery of tests in the mouse. The assays chosen were designed to detect a number of genetic endpoints, namely chromosome breakage, chromosome interchange, and point mutations. Ancillary studies involved germcell survival and reproductive performance. Effects were looked for in both sexes and in a variety of germcell stages. Several of the test systems had been developed at Oak Ridge, and all experiments were carried out there. Exposure to the diesel exhaust took place at the EPA laboratory at Cincinnati. In the maximum-exposure group, the total intake of exhaust per mouse was about 85 times the 30-year (generation-length) intake by a person in an average U.S. environment.

2. OVERALL CONCLUSION

The results of all genetic tests in both sexes were negative. Small but unequivocal effects on the reproductive performance of females of one strain could be observed, consisting of a decrease in the number of ovulations and an increase in the interval between mating opportunity and copulation. It is not known whether these effects were the result of damage directly to the ovary or to some other endocrine organ (e.g., pituitary). There was no detectable effect of diesel exposure on the number and distribution of cell types in the testis.

The absence of genetic effects could indicate either that no active metabolites reached the gonads, or that the germcells have an efficient repair system against induction of mutational events by such metabolites. In experiments with chemical agents, one does not expect -- and, in fact, does not find -- good correlation between transmitted damage induced in mammalian germcells *in vivo* and results from other test systems. It is the former result that is pertinent to transmission of genetic lesions to future generations of human beings; and the work summarized in this report thus indicates that transmitted genetic effects are not a major hazard from exposure to diesel exhaust. The findings reported here must, however, not be used to draw any conclusions concerning possible risks to the exposed individual himself.

3. GENERAL PROCEDURE

All mice for the experiment were bred at Oak Ridge, shipped by air-conditioned van to the EPA laboratory at Cincinnati for exposure to Diesel exhaust or air, and returned to Oak Ridge for the subsequent genetic experiments. The exposure dates are listed separately for the individual projects, but all fell within the period of March 21 to June 4, 1979.

Because of the danger of introducing pathological conditions into the valuable Oak Ridge Mammalian Genetics facility, mice returning from Cincinnati were placed into a quarantine facility in which initial matings were carried out for some of the projects, and the entire procedure took place for others. Animals were monitored for key diseases before their departure to Cincinnati and, again, after their return to the Oak Ridge quarantine building.* The first group to return to Oak Ridge was found to

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*Disease monitoring was under the supervision of the ORNL Biology Division veterinarian, Dr. G. A. Bingham. Tests were performed for 21 organisms and external parasites. These tests were designed to detect the presence of the various organisms with a confidence level of 95% or better, based upon estimates of the prevalence of each organism within the colonies (if present at all). Necropsies were performed on 80 representative mice [(101 x C3H)F_1, T, and SB] before any animals were taken to Cincinnati for exposure. Following return of the first batch, after 5 weeks at Cincinnati, 34 mice were necropsied. Some of the organisms that were present before the mice were sent to Cincinnati were not tested for on their return; i.e., the returning mice were not tested for Polyoma virus, internal parasites, and external parasites. Antibodies to the following three viruses were detected in this batch of mice. Reovirus type 3, Theiler's GDVII and MVM, and Stapholococcus aureus. On the basis of these findings, the animals were not returned to the Oak Ridge Mammalian Genetics facility where they might have infected the valuable colony. From the batches of mice that came back from Cincinnati after about 8 and 11 weeks, respectively, 34 and 20 animals were necropsied and tested as above. Test results revealed no organisms that were not detected in some of the mice before being sent to Cincinnati; or did these mice have the antibodies to Reovirus type 3, Theiler's GDVII, or MVM that were detected in the first returning batch. These groups nevertheless joined the first batch in the separate building and all experiments joined the first batch in the separate building and all experiments were completed there. Disease monitoring was again carried out in December of 1979, when the collection of offspring in the specific-locus experiment was completed and had yielded 3 (surviving) exceptional animals (see Project #1). In order to make the genetic testing of these animals more convenient, we desired to transfer them to the Mammalian Genetics facility. This transfer

carry 3 viruses not present before they were shipped to Cincinnati. We therefore had to utilize a building separate from the Mammalian Genetics facility (as well as from the quarantine building) in which to complete all experiments.

At Cincinnati, mice were housed 3, 4, or 12 animals to a cage,** and cages were placed into 100 cf exposure chambers, having a horizontal cross section of 5 x 5 feet. One of these chambers received CBR-filtered and -conditioned air, while the other chamber was connected by piping to an automobile diesel engine exhaust dilution system. The six-cylinder Nissan engine was operated under load on the Federal Short Cycle, and the exhaust diluted with filtered and conditioned air at the ratio of 1:18. The diesel particulate concentration in the chamber averaged 6 mg/m^3 during the exposure period of 8 hours per day and 7 days of the week. All engine operations, aerometry measurements, and animal care were performed by the EPA staff at Cincinnati. [A few animals were lost during the exposure period (presumably in the process of transfer to clean cages), and a few others during the overnight storage between final removal from the chambers and return shipment to Oak Ridge (when some of the cardboard shipping boxes were chewed through). These numbers are listed separately for each experiment.]

We calculate that, during a 10-week exposure under these conditions, the total intake of exhaust per mouse was about 85 times the 30-year (generation-length) intake by a person in an average U.S. environment (urban-rural). This calculation is based on particulate concentration (mouse, 6 mg/m^3 vs. man 0.3 $\mu g/m^3$), length of exposure (mouse, 10 x 7 x 8/24 = 23.3 days vs. man 30 years = 10,957 days), and pulmonary ventilation rate (mouse 2x man).

Tests were carried out for several types of genetic effects, namely, point mutations in males (specific-locus test), chromosome damage in males (dominant-lethal and heritable translocation tests), and chromosome damage in females (dominant-lethal test). In addition, we looked for reproductive effects in females, and for changes in testis histology. The scope of the experiment did not include chemical determinations of what, if any, substances reached the gonads. If active material from diesel exhaust fails to get into mammalian gonads, this would lead to negative findings in the experiments reported, and presumably also to an absence of risk from genetic lesions transmitted to future generations.

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was effected subsequent to obtaining negative results in the disease monitoring.

** cages of different size

4. REPORTS OF INDIVIDUAL EXPERIMENT

Project #1

TEST FOR HERITABLE POINT MUTATIONS IN MALE MICE

Objectives and relation to other projects

The objective of this project was to test for the induction of transmitted point mutations (intragenic changes and small deficiencies) by means of the specific-locus method -- currently the only practical and reliable method in use in mammals that will detect and quantify this endpoint. This method has recently proved the high sensitivity of in vivo mammalian germcells to chemicals that are only weakly mutagenic (ethylnitrosourea) or nonmutagenic (procarbazine) in bacterial tests or in in vitro systems. Where chemicals that are mutagenic in other tests fail to produce mutations in mammalian spermatogonia, this can be due to unstable metabolites not reaching the target, to cell selection, or to spermatogonial repair systems. Thus, one does not necessarily expect a good correlation between transmitted damage induced in mammalian germcells in vivo and results from other test systems. However, it is the former result that is pertinent to transmission of genetic lesions to future generations of human beings.

In planning the experiment, the number of offspring to be scored was calculated so as to be sufficient either for showing a significantly positive effect, or for ruling out, with a high degree of confidence, that the induced mutation rate, at the level of human exposure (see calculation under Results), could be higher than a small fraction of the spontaneous rate. In order to meet the objectives of the latter alternative, mice had to be exposed to a quantity of Diesel exhaust that was a high multiple of that accumulated by the average American in one human generation.

For assessments of human risk, the stage of prime importance in the male is the spermatogonial stem cell, which can harbor (and transmit) mutations for the lifetime of the individual. The bulk of our data were therefore derived from Diesel exposures to that stage. However, we also obtained enough specific-locus data for the (transitory) postspermatogonial stages to rule out the possibility of a greatly elevated mutational sensitivity during that period.

Since a very large (> 800,000) and reliable historical control exists for spontaneous mutations in specific-locus experiments, we decided to use all our available resources for the study of experimental groups only. A

large sample could therefore be accumulated.

Exposure

Two groups of males were exposed: group A (114 mice) for 5 weeks (3/21-4/24/79) and group B (138 mice) for 10 weeks (3/23-6/4/79). Exposure to the Diesel exhaust was provided for 8 hours per day, 7 days per week, except for an engine shut-down 4/24-4/26, inclusive.

Materials and methods

The exposed males in both groups were (101 x C3H)F_1 or (C3H x 101)F_1 hybrids who were returned to Oak Ridge the day after end of exposure. Immediately upon their return, 86 males from each of groups A and B were mated to 4 females, each, of the multiple-recessive T stock (a/a; b/b; $c^{ch}p$/$c^{ch}p$; d se/d se; s/s). Once a week for the next 4 weeks in group A, and next 5 weeks in group B, the males were shifted to a new set of four females, while the females they left behind were separated one to a cage. In group A, males were killed following these 5 weeks of serial mating, having had the opportunity to mate with altogether 1720 females (20 for each male). In group B, after the first 6 weeks of serial matings (2064 mating opportunities), the males were returned to their first-week mates to repeat the 6-week cycle several more times until early November 1979.

All offspring were observed at about 3 weeks of age for any externally visible changes from the normal phenotype. Exceptionals were saved for further genetic testing, while normals were discarded.

Results

Except for possible slight effects of travel (which resulted in only 81 or 84% as many offspring from first-week than average later-week matings, see Table 1), both groups of males appeared normal with regard to breeding performance, producing average littersizes of 7.11 and 7.09 overall, respectively, and impregnating 75.6% and 76.3% of the females during the first 5 weeks of matings. While we had no concurrent controls, these figures are within the range of the control experience with similar mating regimes (e.g., for an unrelated specific-locus experiment conducted in 1979, average littersize was 6.94 over a 21-week period). Nothing in the breeding performance suggested even a short temporary sterile period which would have been indicative of spermatogonial killing.

The number of offspring examined and exceptionals found are shown in Table 1. Only 4 exceptional animals were found*, and none of these

*The 4 exceptionals were as follows: 2 animals with medium white belly spot, probably resulting from heterozygous expression of s (Note: all offspring of these animals are +/s); one slightly mottled with white, still under test, but not a 7-locus mutant; one, very small and with various anomalies (fused eyelids, scaly tail, shorter ears), died untested at 4 weeks of age.

TABLE 1. RESULTS OF SPECIFIC-LOCUS TEST, FOLLOWING EXPOSURE OF MALE MICE TO DIESEL EXHAUST

Week, after end of exposure, in which matings occurred*	No. of weeks of exposure in following germcell stages†			Numbers of		
	Meiotic and postmeiotic	Differentiating sp'gonia	Sp'gonial stemcells	offspring examined	exceptionals	mutants
Group A						
1st	5	0	0	1556	0	0
2nd	4	1	0	1911	0	0
3rd	3	2	0	1753	0	0
4th	2	2	1	1945	0	0
5th	1	2	2	2075	0	0
Total				9240	0	0
Group B						
1st	5	2	3	1643	0	0
2nd	4	2	4	1802	0	0
3rd	3	2	5	1970	0	0
4th	2	2	6	1923	0	0
5th	1	2	7	2181	0	0
6th	0	2	8	1976	0	0
7th	0	1	9	1866	0	0
8th-20th	0	0	10	19,971	4	0
Total				33,332	4	0
Grand Total				42,572	4	0

*Germcell-stage information (mid-section of the table) applies to matings made at the beginning of the week shown in this column.

†Analysis based on findings of Oakberg (1968) with regard to duration of various germcell stages.

turned out to be due to a mutation at one of the 7 loci. There is no significant difference between 4 exceptionals in 19,971 offspring (8th-20th week) and 0 in 13,361 (1st-7th week).

It will be noted from Table 1 that, except for the 8th through 20th weeks of group B, the exposure was divided differently among the germcell stages for each week's production of offspring. For purposes of analysis, we have grouped the results (Table 2) into offspring derived from germcells that were exposed 3 or more weeks during meiotic and postmeiotic stages ("postspermatogonial" group) and those derived from germcells exposed 8 or more weeks in spermatogonial stages (stemcells or differentiating -- "spermatogonial" group). This grouping omits the results for group A, 4th and 5th weeks.

TABLE 2. SPECIFIC-LOCUS RESULTS GROUPED BY GERM-CELL STAGE

Designation	Weeks of exposure during following stages		Number of	
	meiotic and postmeiotic	spermatogonial (stemcell & diff.)	offspring	mutants
"Postspermatogonial"	3-5	0-7	10,635	0
"Spermatogonial"	0-2	8-10	27,917	0

Calculations have been made with each of these groupings for the ratio of induced to spontaneous rate. Using the upper 95% confidence limit of 0, namely 3.3, we have computed the multiple of the spontaneous rate (43/801,406 or 0.000053655) that would not be exceeded in 97.5% of determinations made at the experimental exposure level. For the "spermatogonial" group, for example, the calculation is as follows

$$\frac{\frac{3.3}{27{,}919} - 0.00053655}{0.000053655} = 1.20$$

Since there is some evidence, both from the absence of a sterile period and the normal testis histology (Project #6), that no spermatogonial killing has occurred, there is little likelihood for a humped dose-response curve. We therefore feel that it is conservative to extrapolate these results to the level of the average human exposure, which is about 1/85 of the 10-week experimental exposure [$(6 \times 10^{3}/3 \times 10^{-1})$ (23.3/10,957) (2/1), see General Procedure]. The experimentally observed zero mutation frequency rules out,

with 97.5% confidence, that, at the level encountered by man, the induced mutation rate could be higher than 0.014 times the spontaneous rate. In the absence of information to the contrary, this extrapolation assumes that the proportion of the total exposure that reaches target cells in the testis is roughly similar in mouse and man.

For the "postgonial" group of 10,635 (as defined above and in Table 2) the multiple calculated from the experiment was 4.8, and the weighted average exposure was roughly 34x average human exposure. The multiple of spontaneous rate, extrapolated to human dose, is thus 0.14.

Conclusion

There is no evidence for the induction of point mutations in spermatogonia or in meiotic and postmeiotic stages. For spermatogonia, the male stage of major importance to risk assessment, the experimentally observed zero frequency rules out, with 97.5% confidence, that, at the level of exposure encountered by man, the induced mutation rate could be higher than 0.01 times the spontaneous rate.

Project #2

TEST FOR INDUCTION OF DOMINANT LETHALS IN MALE MICE

Objectives and relationship to other projects

The effectiveness of diesel exhaust in producing chromosomal aberration effects in germ cells of the male mice was studied using two procedures: the dominant-lethal test (this project) and the heritable translocation test (see report on project #3). Since males were exposed to diesel exhaust for a prolonged period and mated immediately afterwards (see below), all spermatogenic cell stages that are known to be sensitive to dominant-lethal induction were presumably exposed. Dominant-lethal effects were evaluated by analyzing uterine content of unexposed females mated to the exposed or control males. When a sperm carrying a lethal mutation is used in fertilization, the resulting embryo dies before or shortly after implantation.

Exposure

Mice were exposed for 8 hours every day from 3-22-79 to 5-14-79 (7.5 weeks), except during the period of 4-22 to 4-27 due to equipment failure.

Materials and Methods

A total of 216 male T-stock mice, 10-16 weeks old, were sent to Cincinnati for exposure. Half of this number were exposed to diesel exhaust and half served as controls. Immediately after the end of treatment, 102 and 100 mice in the experimental and control groups, respectively, were returned to Oak Ridge. The reason why 14 mice disappeared is not known. Upon their return to Oak Ridge, both control and experimental males were randomized into four groups of 24 each, with each group mated with one of four different stocks of females: (C3H x 101)F_1, (SEC x C57BL)F_1, (C3H x C57BL)F_1, or T-stock. Each male was caged with two females. Females were checked for presence of vaginal plugs every morning for seven days posttreatment. Females that mated were replaced by new ones. Females that mated were killed for uterine analysis 12-15 days after observation of the vaginal plug. The use of four stocks of females was necessary in view of our finding that the yield of dominant-lethal mutations in these stocks may vary markedly owing to differences in the repair capability of the fertilized eggs.

Results

Dominant-lethal data summarized in Table 3 represent the result for all matings that occurred during the first 7 days after the end of exposure.

TABLE 3. TEST FOR INDUCTION OF DOMINANT-LETHAL MUTATIONS IN MALE MICE*

Treatment†	Stock of female mice	Number of mated females‡	Proportion of pregnant females (%)	Total implants per fertile female (avg)	Living embryos per fertile female (avg)	Dead implants/ total implants (%)
Diesel exhaust	(C3H x 101)F_1	85	81	7.8	7.5	4
	(C3H x C57BL)F_1	83	87	10.5	10.1	4
	(SEC x C57BL)F_1	94	80	9.9	9.6	3
	T-stock	89	82	8.8	7.4	16
Control	(C3H x 101)F_1	94	77	7.6	7.4	1
	(C3H x C57BL)F_1	85	84	10.1	9.8	3
	(SEC x C57BL)F_1	83	77	9.6	9.3	3
	T-stock	82	87	8.1	7.2	23

*T-stock males were used in the study.

†Mice were exposed during a 7.5-week period.

‡Males were mated during 7 days after the end of exposure. Tabulated data are from all matings during this period.

These data do not show any indication of dominant-lethal effect. Breakdown of data on a daily basis also did not reveal induced dominant lethality.

Conclusions

Results of the dominant-lethal test indicate that the exposure of male mice to diesel exhaust, as described above, did not induce detectable chromosomal aberration effects in germ cells.

Project #3

TEST FOR INDUCTION OF HERITABLE TRANSLOCATIONS IN MALE MICE

Objectives and relationship to other projects

The effectiveness of diesel exhaust in producing chromosomal aberration effects in germ cells of male mice was studied using two procedures: the heritable-translocation test (this project) and the dominant-lethal test (see report on project #2).

The heritable-translocation method is a sensitive and reliable procedure for measuring the frequency of chromosome breakage and rearrangement (exchange of parts) that is transmitted to the next generation. When a sperm carrying chromosome interchange(s) is used in fertilization, the resulting progeny is heterozygous for the translocation and produces two types of gametes, balanced and unbalanced, in approximately equal proportions. Both types of gametes are capable of fertilization, but the unbalanced gametes result in embryonic lethality. For this reason, most translocation heterozygotes are only about half as productive as normal mice. Heterozygotes for certain types of translocations are incapable of producing sperm. In the heritable-translocation procedure, progeny of treated parents are therefore tested for sterility and "partial sterility." Confirmed sterile and partially sterile progeny are then verified cytologically for presence of a translocation. Thus, the heritable-translocation procedure generates the most meaningful information for evaluating hazards from induced chromosome aberration to human population, because it measures transmissible genetic damage.

Exposure

Mice were exposed for 8 hours every day from 3-22-79 to 4-22-79 (4.5 weeks), either to diesel exhaust or to air.

Materials and Methods

Altogether 160 T-stock males were used in the study. Each of these males was caged with three (SEC x C57BL)F_1 females just prior to shipment to Cincinnati for the purpose of augmenting the number of progeny for estimating the control frequency. This also served to rule out the possibility of preexisting translocations. The parental males were 15-18 weeks old when exposure to diesel exhaust was started. They were randomized in equal numbers into experimental and control groups.

Soon after the last exposure, all animals were shipped back to Oak Ridge (except for two mice from each group, lost at Cincinnati). Each male was caged with two (SEC x C57BL)F_1 females immediately upon arrival. Males were separated from females after one week. All male progeny were weaned and subsequently tested for sterility and partial sterility. All sterile and partially sterile animals were examined cytologically.

Results

Heritable-translocation data are shown in Table 4. We tested 1108 male offspring of matings that occurred before parental males were sent to Cincinnati. Among these 1108, one partially sterile translocation carrier was found. In the contemporary control group (matings made after return from Cincinnati), 358 male progeny were tested. No translocations were found in this group. Thus, the control frequency (sum of the two groups) is $\frac{1}{1466}$.

TABLE 4. INDUCTION OF HERITABLE TRANSLOCATIONS IN MALE MICE WITH DIESEL EXHAUST

Treatment*	Number of progeny tested	Number of translocations
Control	1466	1
Diesel exhaust	358†	0

*Male parents were exposed during a 4.5-week period.

†All offspring were conceived during the 7 days following the end of exposure.

In the experimental group, 350 male progeny were tested - no translocations were found.

The test has an estimated power of better than 95% that it could detect an induced rate of 0.015×10^{-2}.

Conclusions

Results of the heritable-translocation study indicate that the exposure of male mice to diesel exhaust, as described above, did not induce transmissible chromosome exchange in male germ cells. This result is consistent with that of the dominant-lethal test (Project #2), which indicated that there was no induction of chromosomal breakage in male germ cells. Together, the two tests fail to provide evidence of chromosomal aberration effect induced in male mice by the diesel exposure.

Project #4

TEST FOR INDUCTION OF GENETIC EFFECTS AND OOCYTE KILLING IN FEMALES

Objectives and relationship to other projects

The effectiveness of diesel exhaust in producing chromosomal and cytotoxic damages to oocytes was studied by means of total reproductive capacity. The mouse ovary is known to be sensitive to even relatively slight insults from known mutagens. Genetic and/or cytotoxic effects on oocytes in different stages of follicular development were measured by simply counting the offspring produced by exposed and control females over a period of time.

Exposure

Mice were exposed to diesel exhaust for 8 hours every day from 3-22-79 to 5-14-79 (7.5 weeks), except during the period of 4-22 to 4-27 due to equipment failure.

Materials and Methods

Altogether 120 (SEC x C57BL)F_1 females (about 12 weeks old) were shipped to Cincinnati; one-half of these were exposed to diesel exhaust, and the remainder sham exposed. The females were returned to Oak Ridge immediately after the last exposure and caged individually with (C3H x 101)F_1 males upon arrival. Reproductive performance of the females was determined by the size of the litters produced in successive matings during a 195-day posttreatment observation period. Reduced litter sizes may indicate induced dominant-lethal mutations or oocyte killing.

Results

Results are summarized in Table 5. During the observation period, no significant difference between experimental and control groups was observed with respect to the number of litters produced per female and with respect to the size of litters produced at various posttreatment intervals - i.e., there was no significant difference in the total number of young produced during the observation period.

Conclusion

The similarity in long-term reproductive performance (pregnancy rate and littersize) between control females and females exposed to diesel exhaust indicates that the exposure to diesel described above did not induce

TABLE 5. REPRODUCTIVE PERFORMANCE OF FEMALE MICE EXPOSED TO DIESEL EXHAUST

Time after end of treatment (days)	Number of females		Proportion of females bearing litter (%)		Average littersize	
	Diesel exhaust	Control	Diesel exhaust	Control	Diesel exhaust	Control
18-24	45	45	80.0	75.6	10.0	9.3
25-43	45	45	95.6	95.6	11.8	11.6
44-62	45	45	93.3	88.9	11.9	12.0
63-81	45	45	95.6	95.6	12.2	11.8
82-100	45	45	88.9	91.1	11.7	12.5
101-119	45	45	84.4	82.2	12.2	12.3
120-138	45	45	64.4	84.4	12.4	11.9
139-157	45	43	80.0	86.0	11.2	11.9
158-176	45	43	82.2	86.0	10.9	11.1
177-195	45	43	86.7	86.0	10.0	9.6

detectable chromosomal or cytotoxic effects in oocytes of the strain of mice employed in this study. Comparisons with short-term effects on female reproduction, studied in another strain, are discussed under Project #5.

Project #5

TEST FOR DOMINANT-LETHAL INDUCTION IN FEMALE MICE

Objective and relation to other projects

The primary objective of this experiment was to test for induction of chromosomal damages in mature and maturing oocytes. Since the accessibility of the ovary to Diesel metabolites may be very different from that of the testis, this project constituted an important companion to projects No. 1, 2, and 3, which assayed for genetic damage in males. An additional objective of this experiment was to test for any short-term effects of Diesel exhaust on reproductive physiology; long-term effects were tested in project No. 4.

Exposure

Females were exposed to Diesel exhaust at Cincinnati for 8 hours daily, 7 days per week from March 22 through May 14, 1979, except for a shutdown of the Nissan engine during the period of April 24-26, inclusive. The total exposure period, not counting shut-downs, was 7 weeks. The control group was exposed to air in an identical chamber during the same period. There were 54 females in each group. Because of caging problems at Cincinnati, twelve mice escaped between removal from the chambers and transport to Oak Ridge. This left 45 in the experimental (Ex) and 51 in the control (Co) group.

Materials and Method

The exposed females were (101 x C3H)F_1 virgins, 11 1/2 to 13 weeks old at the beginning of exposure. Immediately following their return to Oak Ridge -- one day after end of exposure -- they were caged with (101 x C3H)F_1 males (2 ♀♀:1 ♂) and checked for plugs every morning for the next 7 days. Females were sacrificed usually 14 (occasionally 13 or 15) days after the plug date, corpora lutea were counted, and the uterine contents examined. The 6 females that had not copulated during the 7-day plug-check period were left with their mate for an additional 15 days and then sacrificed. For these females, a plug date was estimated from the stage of development of their embryos.

Results

The top portion of Table 6 shows the mating performance, and the bottom shows the dissection results. There is no significant difference in the percentage of matings resulting in pregnancies. Of the ovulated eggs

(represented by corpora lutea, c.l.), approximately equal proportions resulted in living fetuses, and the distribution among the remaining uterine-content categories was also similar between the Ex and Co groups. There is thus no evidence for the induction of a dominant-lethal effect.

TABLE 6. MATING PERFORMANCE AND RESULTS OF UTERINE DISSECTIONS FOR EXPOSED FEMALES AND CONTROLS

	Experimental		Control	
No. ♀♀ exposed	54		54	
No. ♀♀ caged with oo*	45		51	
No. ♀♀ failing to copulate within 15 days	1 (2.2%)		0	
Non-fertile plugs	3 (6.7%)		3 (5.9%)	
Fertile plugs	41 (91.1%)		48 (94.1%)	
	No.	% of c.l.	No.	% of c.l.
Corpora lutea	384	-	521	-
Live fetuses	298	77.6	418	80.2
Death after day 10 p.c.	0	0	1	0.2
Resorbing	37	9.6	37	7.1
Preimplantation loss	49	12.8	65	12.5
Corpora lutea per female	9.37 ± 0.21		10.85 ± 0.15	

*Difference between No. ♀♀ exposed and No. ♀♀ caged with oo is due to ♀♀ that escaped.

In two parameters, however, clear effects of the Diesel exposure are apparent. (a) There is a significiant difference between Ex and Co groups in mean number of corpora lutea (Table 6, last line), and this difference becomes even more striking when one compares the entire distributions (Table 7 and Fig. 1). (b) The average interval between caging a female with a male and copulation (finding of a plug) is significantly longer, due to a shift in the entire distribution (Table 8 and Fig. 2). In order to determine whether the decrease in corpora lutea might be a function of the delay in mating, we tabulated average number of corpora lutea for each mating interval (Table 9) and found the experimental group to be consistently lower. This indicates that the depression in number of ovulations is not merely the result of mating later than normal.

Discussion and Conclusions

A seven-week exposure to exhaust fumes from a Nissan Diesel engine produced no transmissible chromosomal damage, as measured in a dominant-lethal experiment, in mature and maturing oocytes. However, significant, though slight, effects were produced on female reproductive functions, as expressed

TABLE 7. DISTRIBUTION OF CORPORA LUTEA COUNTS IN EXPOSED FEMALES AND CONTROLS

No. of corpora lutea	% (No.) of females	
	Experimental	Control
7	7.3 (3)	0
8	17.1 (7)	2.1 (1)
9	36.6 (15)	8.3 (4)
10	17.1 (7)	25.0 (12)
11	17.1 (7)	33.3 (16)
12	2.4 (1)	29.2 (14)
13	2.4 (1)	2.1 (1)
Total	(41)	(48)

Mean No. corpora lutea, experimentals, 9.37 ± 0.21

Mean No. corpora lutea, controls, 10.85 ± 0.15

$P < 0.0001$

Figure 1

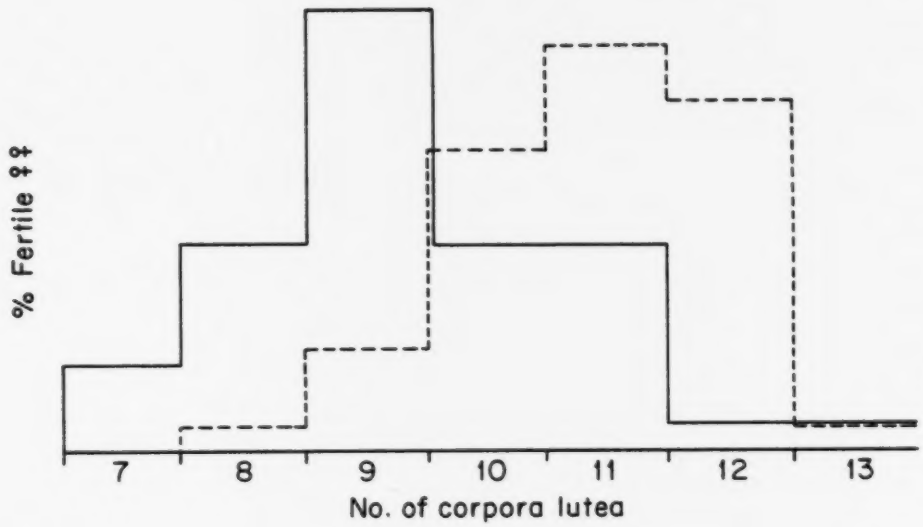

Figure 2

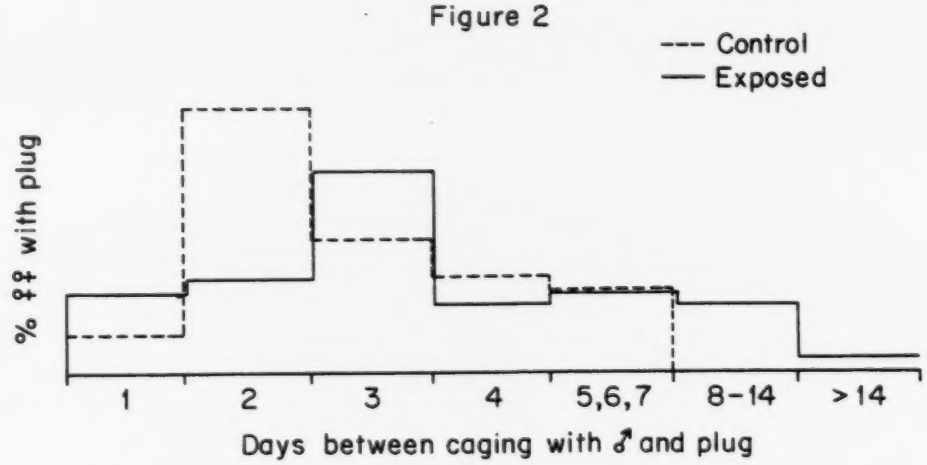

TABLE 8. DISTRIBUTION OF MATING INTERVALS IN EXPOSED FEMALES AND CONTROLS

Interval: caging-to-plug (days)	No. of females	
	Experimental	Control
1	6	3
2	7	22
3	15	11
4	5	8
5	2	5
6	2	1
7	2	1
9	1	0
11	2	0
13	1	0
14	1	0
20*	1	0
Total	45	51

Mean interval, experimental 4.42 ± 0.58

Mean interval, control 2.94 ± 0.18

P = 0.016

*A 20-day interval was arbitrarily assigned for one female who showed no implants 22 days after being caged with male. Minimum interval could have been 16 days. If a smaller interval than 20 days is assigned, significance increases slightly; e.g., for 15 days, P = 0.014.

TABLE 9. AVERAGE NUMBER OF CORPORA LUTEA AT DIFFERENT MATING INTERVALS

Interval: caging-to-plug (days)	Average No. of corpora lutea	
	Experimental	Control
1	8.8 (5)*	10.0 (2)
2	10.3 (6)	10.8 (21)
3	9.6 (14)	11.2 (11)
4	9.2 (5)	10.6 (7)
5, 6, 7	9.0 (6)	11.0 (7)
8 - 14	8.8 (5)	

*No. of females on which the average is based is shown in parentheses.

in a decreased number of ovulations (14% overall) and a lengthening (by about 1.5 days) of the time interval between mating opportunity and copulation.

The results of this study, which concerns matings made mostly within a week after the end of exposure, may be compared with first-litter results for Project #4 (births 18-24 days after end of treatment). The females used in these two projects, though of the same age, were genetically different: (SEC x C57BL)F_1 in Project 4 and (101 x C3H)F_1 in Project 5. For the first-week period, only 75.6% of mated control females produced litters in Project #4, but 100% of controls got pregnant in Project #5. It is therefore difficult to predict whether a 1.5-day (average) diesel-induced lengthening in time interval between mating opportunity and copulation (shift in mean from 2.9 to 4.4 days), such as was determined through day-by-day analysis in Project 5, would have been detectable in Project #4. It seems probable that the method of Project #4 was not sensitive enough to detect such a shift, had it been induced in the (SEC x C57BL)F_1 females. Project #4 should, however, have detected a 16% reduction in average littersize, such as appears to have been induced in Project #5. We conclude that there is a genetic difference in sensitivity of females to the physiological damage(s) responsible for reducing number of ovulated eggs.

Project #6

TEST FOR EFFECTS ON SPERMATOGONIAL SURVIVAL IN THE MOUSE

Objective

Differentiating spermatogonia of the mouse are extremely sensitive to cytotoxic agents such as radiation (1) and chemicals, and often show a positive response even when no genetic damage is observed (2). In the latter case, they provide a sensitive test system to determine if test materials reach the gonads. The response observed, however, is a somatic one (i.e. cell death) and is not necessarily associated with heritable effects. Killing of spermatogonia can, of course, have an effect on fertility.

Materials and Methods

Hybrid male mice of the JH and H strains were exposed to diesel exhaust 8 hrs per day, seven days a week, for 5 and 10 weeks. The mice then were returned to Oak Ridge and killed immediately. Contemporary controls were transported to Cincinnati, handled similarly but exposed to air only, returned to Oak Ridge, and killed at the same time as exposed mice. (Controls for the 10-week group inadvertently were returned one week too early).

Mice were killed by cervical dislocation. The testes were dissected free of connective tissue and fat and fixed in Zenker-formol. Five-micron paraffin sections were stained in periodic-acid-shiff and hematoxylin. One hundred tubule cross sections, distributed among the stages of the seminiferous epithelium on the basis of the frequency distribution of stages of the cycle in controls, were scored for each mouse. Eight spermatogonial classes (A_s, A_{pr} + A_{al}, A_1, A_2, A_3, A_4, In, B) and preleptotene spermatocytes were enumerated. Three controls and 3 treated males were used for each strain and exposure time.

Results

No effect of exposure to diesel exhaust on number spermatogonia or preleptotene spermatocytes was observed for either strain or exposure group. Control counts were the same at 5 and 10 (actually, 9) weeks, so controls were combined by strain. Data are given in Table 10. Differences in cell counts all were within the limits normally observed for control mice.

TABLE 10. MEAN NUMBER OF SPERMATOGONIA AND PRELEPTOTENE SPERMATOCYTES OBSERVED AFTER EXPOSURE TO DIESEL EXHAUST FOR 5 AND 10 WEEKS

Cell Type		JH mice				H mice			
		Control*	Treated			Control*	Treated		
			5 wks.†	10 wks.†	5 & 10 weeks*		5 wks.†	10 wks.†	5 & 10 weeks*
As	Sp'gonia	52.8‡	59.0	45.6	52.3	59.2	57.0	46.3	51.7
Apr + Aal	Sp'gonia	14.2	13.3	9.0	11.2	12.5	13.3	12.0	12.7
A_1	Sp'gonia	73.2	96.7	80.0	88.3	85.0	85.3	81.0	83.2
A_2	Sp'gonia	26.3	21.7	19.7	20.7	29.2	35.3	24.0	29.7
A_3	Sp'gonia	39.8	33.7	41.3	37.5	42.3	40.0	33.0	36.5
A_4	Sp'gonia	82.7	86.0	78.7	82.3	76.3	80.3	68.7	74.5
In	Sp'gonia	137.5	128.3	138.0	133.2	126.5	123.3	124.7	124.0
B	Sp'gonia	243.2	271.0	282.7	276.8	236.8	268.3	255.0	261.7
Pre-lept	Sp'cytes	1129.2	1184.0	1117.7	1150.8	1106.8	1089.7	1079.0	1084.3

*Mean of 6 mice.

†Mean of 3 mice.

‡Number of cells scored in 100 tubule cross sections per mouse, distributed among the stages of the cycle of the seminiferous epithelium on the basis of the frequency distribution in control mice.

Conclusion

Exposure to diesel exhaust had no effect on the number of spermatogonia or preleptotene spermatocytes of mice after 5 or 10 weeks' exposure. Likewise, no irregularities in the normal dynamics of the seminiferous epithelium were observed; cellular associations were the same as in controls, and division of the different spermatogonial types occurred at the normal times in the cycle of the seminiferous epithelium.

1. Oakberg, E. F. Gamma-ray sensitivity of spermatogonia of the mouse. J. Exp. Zool. 134:343-356, 1957.

2. Oakberg, E. F. Response of spermatogonia of the mouse to hycanthone: A comparison with the effect of gamma rays. In, Physiology and Genetics of Reproduction, Part A. E. M. Coutinho and F. Fuchs, eds. Plenum Pub. Corp., N.Y. pp. 197-207.